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OSTEOPROTEGERIN

Field of the Invention

The invention relates generally to polypeptides involved in the regulation of bone metabolism. More particularly, the invention relates to a novel polypeptide, termed osteoprotegerin, which is a member of the tumor necrosis factor receptor superfamily. The polypeptide is used to treat bone diseases characterized by increased bone loss such as osteoporosis.

Background of the invention

Polypeptide growth factors and cytokines are secreted factors which signal a wide variety of changes in cell growth, differentiation, and metabolism, by specifically binding to discrete, surface bound receptors. As a class of proteins, receptors vary in their structure and mode of signal transduction. They are characterized by having an extracellular domain that is involved in ligand binding, and cytoplasmic domain which transmits an appropriate intracellular signal. Receptor expression patterns ultimately determine which cells will respond to a given ligand, while the structure of a given receptor dictates the cellular response induced by ligand binding. Receptors have been shown to transmit intracellular signals via their cytoplasmic domains by activating protein tyrosine, or protein serine/threonine phosphorylation (e.g., platelet derived growth factor receptor (PDGFR) or transforming growth factor -β receptor -I (TGFβR-I), by stimulating G-protein activation (e.g., β-adrenergic Receptor), and by modulating associations with cytoplasmic signal transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNFα. (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNFα binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNFα produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNFα is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNFα induces toxic shock and widespread tissue necrosis. Because of this, TNFα may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-1 receptors, and antibodies that bind TNFα, have been tested for their ability to neutralize systemic TNFα (Loetscher et al. Cancer Cells 3(6), 221-226

(1991)). A naturally occuring form of a secreted TNFR-1 mRNA was recently cloned, and its product tested for its ability to neutralize TNFα activity in vitro and in vivo (Kohno et al. PNAS USA 87, 8331-8335 (1990)). The ability of this protein to neutralize TNFα suggests that soluble TNFα receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR- bearing cells.

An object of the invention to identify new members of the TNFR super family. It is anticipated that new family members, may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely related to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2 related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a fetal rat intestinal cDNA library. A full-length cDNA clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bones density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by the cDNA is termed osteoprotegerin and plays a role in promoting bone accumulation.

The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of osteoprotegerin. Nucleic acids which hybridize to nucleic acids encoding mouse, rat or human Osteoprotegerin as shown in Figures 2B, 9A and 9B are also provided. Preferably, osteoprotegerin

is mammalian osteoprotegerin and more preferably is human osteoprotegerin. Recombinant vectors and host cells expressing osteoprotegerin are also encompassed as are methods of producing recombinant osteoprotegerin. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

Methods of treating bone diseases are also provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone, and bone loss due to rheumatoid arthritis or osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising osteoprotegerin nucleic acids and polypeptides are also encompassed.

Description of the Figures

Figure 1. A. FASTA analysis of novel EST LORF. Shown is the deduced FRI-1 amino acid sequence aligned to the human TNFR-2 sequence. B. Profile analysis of the novel EST LORF shown is the deduced FRI-1 amino acid sequence aligned to the TNFR-profile. C. Structural view of TNFR superfamily indicating region which is homologous to the novel FRI-1.

Figure 2. Structure and sequence of full length rat Osteoprotegerin gene, a novel member of the TNFR superfamily. A. Map of pMOB-B1.1 insert. Box indicates position of LORF within the cDNA sequence (bold line). Black box indicates signal peptide, and gray ellipses indicate position of cysteine-rich repeat sequences. B. Nucleic acid and protein sequence of the Rat Osteoprotegerin cDNA. The predicted signal

peptide is underlined, and potential sites of N-linked glycosylation are indicated in bold, underlined letters. C. Pileup sequence comparison (Wisconsin GCG Package, Version 8.1) of Osteoprotegerin with other members of the TNFR superfamily.

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat Osteoprotegerin protein sequence.

Figure 4. mRNA expression patterns for the Osteoprotegerin cDNA in human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (left two panels), or with the human cDNA insert (right panel).

Figure 5. Creation of transgenic mice expressing the Osteoprotegerin cDNA in hepatocytes. Northern blot expression of HE-Osteoprotegerin transgene in mouse liver.

Figure 6. Increase in bone density in Osteoprotegerin transgenic mice. Panel A. Control Mice. Panel B, Osteoprotegerin expressing mice. At necropsy, all animals were radiographed and photographs prepared. In A, the radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the Osteoprotegerin (B) animals have a poorly defined cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in Osteoprotegerin transgenic mice. Plate A. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power images of the

femurs are shown (Masson Trichrome stain). Stains for tartrate resistant acid phosphatase (TRAP) demonstrate osteoclasts (see arrows) both resorbing cartilage (C) and trabecular bone (D). Note the flattened appearance of osteoclasts on trabecular bone. Plate B. Representative photomicrographs of bones from Osteoprotegerin-expressing animals. In A and B, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the trabecular bone has not been resorbed resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based on TRAP stains, these animals do have osteoclasts (see arrows) at the growth plate (panel C), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of osteoclasts (D), a finding that stands in direct contrast with the control animals (see Plate A, Panal D).

Figure 8. HE-Osteoprotegerin expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due to a point mutation in the M-CSF gene. This results in a marked deficit of circulating and tissue based macrophages. The peripheral blood of Osteoprotegerin expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages, immnohistochemistry was performed using F480 antibodies, which recognize a cell surface antigen on murine macrophages. Panels A and C show low power (4X) photomicrographs of the spleens from normal

and CR1 overexpressors. Note that both animals have numerous F480 positive cells. Monocyte-macrophages were also present in the marrow of normal (B) and HE-Osteoprotegerin overexpressors (D) (40X).

Figure 9. Structure and sequence of mouse and human Osteoprotegerin cDNA clones. A. Mouse cDNA and protein sequence. B. Human cDNA and protein sequence. The predicted signal peptides are underlined, and potential sites of N-linked glycosylation are indicated in bold. C. Sequence alignment and comparison of rat, mouse and human Osteoprotegerin amino acid sequences.

Figure 10. Expression and secretion of full length and truncated mouse Osteoprotegerin-Fc fusion proteins. A. Map indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of and SDS-polyacrylamide gel of conditioned media obtained from Fl.Fc (Full length Osteoprotegerin fused to Fc at Leucine 401) and CT.Fc (Carboxy-terminal truncated osteoprotegerin fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of conditioned media obtained from Fl.Fc and CT.Fc fusion protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line.

Figure 11. Expression of human Osteoprotegerin in E. coli. A. Construction of a bacterial expression vector. The LORF of the human Osteoprotegerin gene was amplified by PCR, then joined to a oligonucleotide linker fragment, and ligated into pAMG21 vector DNA. The resulting vector is capable of expressing

Osteoprotegerin residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the pAMG21-human Osteoprotegerin -32-401 plasmid. Lane 1, MW standards; lane 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole cell lysate; lane 8, purified inclusion bodies obtained from whole cell lysate.

Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor superfamily was identified as an expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library . The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2A, 9A and 9B, respectively. All three sequences showed strong similarity to the extraceullular domains of TNFR family members. None of the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human genes spanning nucleotides 1200-1353 shown in Figures 9B was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat, mRNA expression was detected in kidney, liver, placenta and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the

same tissues along with lymph node, thymus, spleen and appendix.

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of bone and cartilage. A decrease in the number of trabecular osteoclasts in the bones of Osteoprotegerin expressor animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in transgenic expressors, the TNFR-related proteins described herein are termed osteoprotegerins.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse Osteoprotegerin in 293 cells and human osteoprotegerin in E. coli is described in Examples 6 and 7. Mouse Osteoprotectin was produced as an Fc fusion which was purified by Protein A affinity chromatography.

Osteoprotegerin may be important in regulating bone resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor-ligand interaction involved in the osteolytic pathway. One aspect of the regulation appears to be a reduction in the number of osteoclasts.

Nucleic Acids

The invention provides for an isolated nucleic acid encoding a polypeptide having at least one

of the biological activities of osteoprotegerin. As described herein, the biological activities of Osteoprotegerin include, but are not limited to, any activity involving bone metabolism and in particular, include increasing bone density. The nucleic acids of the invention are selected from the following:

a) the nucleic acid sequences as shown in Figures 2B, 9A and 9B or complementary strands thereof;

b) the nucleic acids which hybridize under stringent conditions with the polypeptide-encoding region in Figures 2B, 9A and 9B; and

c) nucleic acids which hybridize under stringent conditions with nucleotides 148 through 337 inclusive as shown in Figure 2B.

d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

The invention provides for nucleic acids which encode rat, mouse and human Osteoprotegerin as well as nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological activities of Osteoprotegerin. Also provides for nucleic acids which hybridize to a rat osteoprotegerin EST encompassing nucleotides 148-337 as shown in Figure 2B. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C described in Example 1 of the specification. Equivalent stringency to these conditions may be readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding Osteoprotegerin-related polypeptides which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the nucleic acids are as shown in Figures 2A, 9A and 9B.

DNA encoding rat osteoprotegerin was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on _____ under ATCC accession no. _____. DNA encoding mouse Osteoprotegerin was provided in plasmid pRcCMV-murine Osteoprotegerin deposited with the American Type Culture Collection, Rockville, MD on _____ under accession no. _____. DNA encoding human Osteoprotegerin was provided in plasmid pRcCMV - human Osteoprotegerin deposited with the American Type Culture Collection, Rockville, MD on _____ under accession no. _____. Further, the nucleic acids of the invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. _____, _____ , and _____ and have at least one of the biological activities of osteoprotegerin.

Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2A, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution insertion or deletion of one or more residues such that the resulting sequences encode polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of Osteoprotegerin. The nucleic acid derivatives may be naturally occurring, such as by splice variation or polymorphism, or may be constructed using site-directed mutagenesis techniques available to the skilled worker. It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of Osteoprotegerin having an extracellular domain as shown in Figures 2B, 9A and 9B along with transmembrane and cytoplasmic domains.

Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing Osteoprotegerin. In humans, tissue sources for Osteoprotegerin include kidney, liver, placenta and heart. Genomic DNA encoding Osteoprotegerin is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by procaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of Osteoprotegerin sequences in biological samples in order to determine which cells and tissues are expressing Osteoprotegerin mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to Osteoprotegerin. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologus sequences. The nucleic acids are also useful for modulating the expression of Osteoprotegerin levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding Osteoprotegerin, host cells transformed with said vectors and methods for the production of Osteoprotegerin are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185 (Goeddel, D.V. ed.) Academic Press (1990).

Host cells for the production of Osteoprotegerin include procaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression. Preferred mammalian host cells include COS, CHOd-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for Osteoprotegerin activity. Mammalian expression allows for the production of secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of Osteoprotegerin contain at a minimum sequences required for vector propogation and for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of Osteoprotegerin are also included. Such vectors include promoters which function specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of Osteoprotegerin in targeted human cells.

Using an appropriate host-vector system, Osteoprotegerin is produced recombinantly by culturing a host cell transformed with an expression vector containing nucleic acid sequences encoding Osteoprotegerin under conditions such that Osteoprotegerin is produced, and isolating the product of expression. Osteoprotegerin is produced in the supernatant of transfected mammalian cells or in inclusion bodies of transformed bacterial host cells. Osteoprotegerin so produced may be purified by procedures known to one skilled in the art as described below. The expression of Osteoprotegerin in mammalian and bacterial host systems is described in Example 6 and 7. It is anticipated that the specific plasmids and host cells described are for illustrative purpose and that other available plasmids and host cells could also be used to express the polypeptides.

The invention also provides for expression of Osteoprotegerin from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of Osteoprotegerin from the host chromosome. See U.S. Patent No. ___ which describes regulation of endogenous expression of erythropoietin.

Polypeptides

The invention provides for Osteoprotegerin, a novel member of the TNF receptor superfamily, having an activity associated with bone metabolism and in particular having the activity of inhibiting bone resorption thereby increasing bone density. Osteoprotegerin refers to a polypeptide having an amino acid sequence of mouse, rat or human Osteoprotegerin or a derivative thereof having at least one of the biological activities of Osteoprotegerin. The amino acid sequences of rat, mouse and human osteoprotegerin are shown in Figures 2A, 9A and 9B respectively. A

derivative of Osteoprotegerin refers to a polypeptide having an addition, deletion, insertion or substitution of one or more amino acids such that the resulting polypeptide has at least one of the biological activities of Osteoprotegerin. The biological activities of Osteoprotegerin include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have the amino terminal leader sequence of 21 amino acids removed.

Osteoprotegerin polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO: __ (rat), SEQ ID NO: __ (mouse) and SEQ ID NO: __ (human). Also encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of Osteoprotegerin; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of Osteoprotegerin, in particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain.

Modifications of Osteoprotegerin polypeptides are encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-

terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Further modifications of Osteoprotegerin include chimeric proteins wherein Osteoprotegerin is fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence which allows the resulting fusion protein to retain the activity of Osteoprotegerin. The heterologous sequences include for example, immunoglobulin fusions, such as Fc fusions, which may aid in purification of the protein.

The polypeptides of the invention are isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing Osteoprotegerin, or purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association with other human proteins, such as the expression product of a bacterial host cell.

Also provided by the invention are chemically modified derivatives of osteoprotegerin which may provide additional advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

A method for the purification of Osteoprotegerin from natural sources and from transfected host cells is also included. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-Osteoprotegerin antibody or biotin-streptavidin affinity complex and the like.

Antibodies

Also encompassed by the invention are antibodies specifically binding to Osteoprotegerin. Antigens for the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the Osteoprotegerin sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with Osteoprotegerin are known to one skilled in the art (see, for example, Harlow and Lane Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the

art. Also encompassed are human antibodies made in mice.

Anti-osteoprotegerin antibodies of the invention may be used as an affinity reagent to purify Osteoprotegerin from biological samples. In one method, the antibody is immobilized on CnBr-activated Sepharose and a column of antibody-Sepharose conjugate is used to remove Osteoprotegerin from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate Osteoprotegerin in biological samples by methods described below.

Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the polypeptide of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascrobic acid or sodium metabisulfite. Also encompassed are compositions comprising Osteoprotegerin modified with water soluble polymers to increase solubility or stability. Compositions may also comprise incorporation of Osteoprotegerin into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Selection of a particular composition will depend upon a number of

factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the Osteoprotegerin coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes, osteoblasts and osteoclasts, are involved in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal

tissue and in resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of Osteoprotegerin. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with Osteoprotegerin is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with osteoprotegerin include the following:

Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathryoidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Paget's disease of bone (osteitis deformans) in adults and juveniles

Osteomyelitis, or an infectious lesion in bone, leading to bone loss.

Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignacies

(multiple myeloma, lymphoma and leukemia), idiopathic hypercalcemia, and hypercalcemia associated with hyperthryoidism and renal function disorders.

Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases.

Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions.

Bone loss due to rheumatoid arthritis.

Periodontal bone loss.

Osteolytic metastasis

It is understood that Osteoprotegerin may be used alone or in conjunction with other factors for the treatment of bone disorders. In one embodiment, osteoprotegerein is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12, transforming growth factor -β (TGF-β) and TGF-β family members, interleukin-1 inhibitors, TNFα inhibitors, parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof, E series prostaglandins, bisphosphonates (such as alendronate and others), and bone-enhancing minerals such as fluoride and calcium.

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

EXAMPLE 1

Identification and isolation of the rat osteoprotegerin cDNA

Materials and method for cDNA closing and analysis are described in Maniatis et al. Molecular Cloning, 2d ed., CHSL Press (1989). A cDNA library was constructed using mRNA isolated from embryonic d20 intestine for EST analysis (Adams et al. Science 252: 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A random primed cDNA library was prepared using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md). The random cDNA primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5'-AAAGGAAGGAAAAAAGCGGCCGCTACANNNNNNNNT-3'

Not I

For the first strand synthesis three separate reactions were assembled that contained 2.5 ug of poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:choroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA products of the three reactions were combined and ligated to the following ds oligonucleotide adapter:

5'-TCGACCCACGCGTCCG-3'
3'- GGGTGCGCAGGCp-5'

After ligation the cDNA was digested to completion with Not I, extracted with phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer. The two fractions containing the largest cDNA products were pooled, ethanol precipitated and then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann et al, 1991). The ligated cDNA was introduced into competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented LB nutrient media. The colonies that arose were picked and arrayed onto 96 well microtiter plates containing 200 µl of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp.,Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 ul of diluted bacterial cultures were added to PCR reaction

mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5'-TGTAAAACGACGGCCAGT-3'
5'-CAGGAAACAGCTATGACC-3'

The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions: 94 C for 2 minutes; 30 cycles of 94 C for 5 seconds, 50 C for 5 seconds, and 72 C for 3 minutes.; 72 C for 4 minutes. After incubation in the thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'-CAATTAACCCTCACTAAAGG-3') Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures.

The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson et al. Meth. Enzymol. 183, (1990)). Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. Cell 76, 959-962 (1994)), using the sequence profile method of Gribskov et al. (PNAS USA 83, 4355-4359 (1987), as modified by Luethy et al. (Protein Science 3, 139-146 (1994)).

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a possible new member of the TNFR superfamily. FRI-1 contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-2). The region compared showed an ~43% homology between TNFR-2 and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR superfamily yielded a Z score of ~8, indicating that the FRI-1 gene possibly encodes a new family member.

To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

5'-GCATTATGACCCAGAAACCGGAC-3'
5'-AGGTAGCGCCCTTCCTCACATTC-3

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cycler with the following cycle conditions: 2 min at 94°c,1 cycle; 15 sec at 94°c, then 45 sec at 65°c, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto

sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a ^{32}P-dCTP labelled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C. Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 ug/ml denatured salmon sperm DNA, and ~5 ng/ml of labelled probe for ~18 hours at 42°C. The filters were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C. Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA search of the public database to detect any existing sequence matches and/or similarities. No matches to any known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-2 genes. A methionine start codon is found at bp 124 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches, although there appeared to be a strong

similarity to many members of the TNFR superfamily, most notably the human and mouse TNFR-2. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and then modified by PrettyPlot (Wisconsin GCG package, version 8.1). This alignment shows a clear homology between the full length FRI-1 gene product and all other TNFR family members. The homologus region maps to the extracellular domain of TNFR family members, and corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted receptor, similar to TNFR-1 derived soluble receptors (Kohno et al. PNAS USA 87, 8331-8335 (1990)). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin.

EXAMPLE 2

Osteoprotegerin mRNA Expression Patterns in Tissues

Multiple human tissue northern blots (Clonetech) were probed with a 32P-dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to determine patterns of expression. Northern blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's solution, 0.1% SDS, 100 ug/ml denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for

10 min at RT, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

Using a probe derived from the rat gene, a predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest levels are detected in the kidney. A large mRNA species of Mr 4.5 and 7.5 kb was detected in skeletal muscle and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA. Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In addition, relatively high levels of the 2.4 kb transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript detected by both the rat and human Osteosprotegerin gene is almost identical to the length of the rat pB1.1 FRI-1 insert, suggesting it was a full length cDNA clone.

EXAMPLE 3

Systemic delivery of Osteoprotegerin in transgenic mice

The rat Osteoprotegerin clone pB1.1 was used as template to PCR amplify the coding region for subcloning into an ApoE-liver specific expression vector (Simonet et al. J. Clin. Invest. 94, 1310-1319 (1994), and PCT Application No. US94/11675 and co-owned U.S. Serial No. 08/221,767. The following 5' and 3' oligonucleotide primers were used for PCR amplification, respectively:

5'-GACTAGTCCCACAATGAACAAGTGGCTGTG-3'
5'-ATAAGAATGCGGCCGCTAAACTATGAAACAGCCCAGTGACCATTC-3'

The PCR reaction mixture (Boehringer-Mannheim) was treated as follows: 94°C for 1 minute, 1 cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1 minute, 25 cycles. Following amplification, the samples were purified over Qiagen PCR columns and digested overnight with SpeI and NotI restriction enzymes. The digested products were extracted and precipitated and subcloned into the ApoE promoter expression vector. Prior to microinjecting the resulting clone, HE-Osteoprotegerin, it was sequenced to ensure it was mutation-free.

The HE-Osteoprotegerin plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid DNA was digested with XhoI and Ase I, and the 3.6 kb transgene insert was purified by gel electrophoresis. The purified fragment was diluted to a stock injection solution of 1 ug/ml in 5 mM Tris, pH 7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x BDF1-bred mice were injected essentially as described (Brinster et al., PNAS USA 82, 4338 (1985)), except that injection needles were beveled and siliconized before use. Embryos were cultured overnight in a CO_2 incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3'

5'- CGC CGT GTT CCA TTT ATG AGC-3'

The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic founders.

At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls (1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the remaining 4 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as described (McDonald et al. Meth. Enzymol. 152, 219 (1987)). Northern blot analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden et al. Meth. Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with 32P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 ug/ml denatured salmon sperm DNA and 2-4 x 10^6 cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33,and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since

Osteoprotegerin is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

EXAMPLE 4

Biological activity of Osteoprotegerin

Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures: Prior to euthanasia, all animals had their identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was performed just after terminal anesthesia by lethal CO2 inhalation, and prior to the gross dissection. Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, eosphagus, thyroid, jejunem, cecum, rectum, adrenals, urinary bladder, and skeletal muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals, spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3 um sections were obtained. Bone tissue was decalcified using a formic acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with

Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate resistant acid phosphatase (TRAP), an enyzme highly expressed by osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage. Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect replicating cells and cells of the monocyte-macrophage lineage, respectively. To detect F480 surface antigen expression, formalin fixed, paraffin embedded 4μm sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated strepavidin (BioGenex San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin.

Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The relative size of femurs in the

expressors were not different from the the control mice.

Histological analysis of stained sections of bone from the Osteoprotegerin expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the Osteoprotegerin-induced skeletal changes were systemic. The residual bone marrow showed predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-macrophage derivation in the mouse, showed the presence of F480 positive cells in the marrow spaces. Focally, flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

The mesenchymal cells lining the bony trabeculae were flattened and appeared inactive. Based on H&E and TRAP stains, osteoclasts were rarely found on the trabecular bone surfaces in the Osteoprotegerin expressors. In contrast, osteoclasts and/or chondroclasts were seen in the region of the growth plate resorbing cartilage, but their numbers may be reduced compared to controls. Also, osteoclasts were present on the cortical surface of the metaphysis where modelling activity is usually robust. The predominant difference between the expressors and controls was the profound decrease in trabecular osteoclasts, both in the vertebrae and femurs. The extent of bone accumulation was directly correlated with the level of

Osteoprotegerin transgene mRNA detected by northern blotting of total liver RNA.

The spleens from the Osteoprotegerin expressors had an increased amount of red pulp with the expansion due to increased hematopoiesis. All hematopoietic lineages are represented. F480 positive cells were present in both control and Osteoprotegerin expressors in the red pulp. Two of the expressors (2 and 17)had foci of extramedullary hematopoiesis within the liver and this is likely due to the osteopetrotic marrow.

There were no observable abnormalities in the thymus, lymph nodes, gastrointestinal tract, pancreato-hepatobiliary tract, respiratory tract, reproductive system, genito-urinary system, skin, nervous system, heart and aorta, breast, skeletal muscle and fat.

EXAMPLE 5

Isolation of mouse and human Osteoprotegerin cDNA

A cDNA clone corresponding to the 5' end of the mouse Osteoprotegerin mRNA was isolated from a mouse kidney cDNA library (Clontech) by PCR amplification. The oligonucleotides were derived from the rat Osteoprotegerin cDNA sequence and are shown below:

5'-ATCAAAGGCAGGGCATACTTCCTG-3'
5'-GTTGCACTCCTGTTTCACGGTCTG-3'

5'-CAAGACACCTTGAAGGGCCTGATG-3'
5'-TAACTTTTACAGAAGAGCATCAGC-3'

5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3'
5'-AGCTCTAGAGAAACAGCCCAGTGACCATTCC-3'

The partial and full-length cDNA products obtained in this process were sequenced. The full-length product was digested with Not I and Xba I, then directionally cloned into the plasmid vector pRcCMV (Invitrogen). The resulting plasmid was named pRcCMV-Mu-Osteoprotegerin. The nucleotide sequence of the cloned product was compared to the rat Osteoprotegerin cDNA sequence. Over the 1300 bp region spanning the Osteoprotegerin LORF, the rat and mouse DNA sequences are approximately 88% identical. The mouse cDNA sequence contained a 401 aa LORF, which was compared to the rat Osteoprotegerin protein sequence and found to be ~94% identical without gaps. This indicates that the mouse cDNA sequence isolated encodes the murine Osteoprotegerin protein, and that the sequence and structure has been highly conserved throughout evolution. The mouse Osteoprotegerin protein sequence contains an identical putative signal peptide at its N-terminus, and all 4 potential sites of N-linked glycosylation are conserved.

A partial human Osteoprotegerin cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'
5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'

This PCR product was sequenced and used to design primers for amplifying the 3' end of the human cDNA using ahuman osteoprotegerin genomic clone in lambda as template:

5'-TCCGTAAGAAACAGCCCAGTGACC-3'
5'-CAGATCCTGAAGCTGCTCAGTTTG-3'

The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design 5' and 3' human-specific primers useful for amplifying the entire human Osteoprotegerin cDNA coding sequences:

5'-AGCGCGGCCGCGGGGACCACAATGAACAAGTTG-3'
5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3'

The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human Osteoprotegerin. The nucleotide sequence of the cloned product was compared to the rat and mouse Osteoprotegerin cDNA sequences. Over the 1300 bp region spanning the Osteoprotegerin LORF, the rat and mouse DNA sequences are approximately 78-88% identical to the human Osteoprotegerin cDNA. The human Osteoprotegerin cDNA sequence also contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human Osteoprotegerin protein is approximatlely 85% identical, and ~90% identical to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked glycosylation, 4 of which are conserved between the rat and mouse Osteoprotegerin proteins.

EXAMPLE 6

Production of recombinant secreted Osteoprotegerin protein in mammalian cells

To determine if Osteoprotegerin is actually a secreted protein we expressed the mouse cDNA, fused to

the human IgG1 Fc domain as a tag (Capon et al. Nature 337, 525-531 (1989)), in human 293 fibroblasts. The cloned mouse cDNA was amplified using the following two sets of primer pairs:

Pair 1
5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3'
5'-CCTCTGCGGCCGCTAAGCAGCTTATTTTCACGGATTGAACCTG-3'

Pair 2
5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3'
5'-CCTCTGCGGCCGCTGTTGCATTTCCTTTCTG-3'

The first pair amplifies the entire Osteoprotegerin LORF, and creates a Not I restriction site which is compatable with the in-frame Not I site Fc fusion vector FcA3. FcA3 was prepared by engineering a Not 1 restriction site 5' to aspartic acid reside 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the Osteoprotegerin protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all 401 Osteoprotegerin residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc).
The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of Osteoprotegerin, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artifical Not I restriction site which fuses the C-terminal truncated Osteoprotegerin LORF at position $Threonine_{180}$ directly to the IgG1 Fc domain (CT.Fc).

Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is

capable of episomal replication in 293-EBNA-1 cells. The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 μg/ml hygromycin to select for vector expression, and the resulting drug-resistant mass cultures were grown to confluence. The cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by the drug resistant 293 cultures. In the pCEP4-F1.Fc and the pCEP4-CT.Fc conditioned media, unique band of the predicted size were abundantly secreted. The full-length Fc fusion protein accumulated to a high concentration, indicating that it may be stable. Both Fc fusion proteins were detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant Osteoprotegerin products.

The full length Osteoprotegerin-Fc fusion protein was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read after 19 cycles:

NH_2-E T L P P K Y L H Y D P E T G H Q L L-CO_2H

This sequence was identical to the predicted mouse Osteoprotegerin amino acid sequence beginning at amino acid residue 22, suggesting that the natural mammalian leader cleavage site is between amino acid residues Q_{21}-E_{22}, not between Y31-D32 as originally predicted. The expression experiments performed in

293-EBNA cells with pCEP4-Fl.Fc and pCEP4-CT.Fc demonstrate that Osteoprotegerin is a secreted protein, and may act systemically to bind its unidentified ligand.

EXAMPLE 7

Expression of human Osteoprotegerin in E. coli

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120. This host has the $lacI^Q$ promoter and lacI gene integrated into a second site in the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human Osteoprotegerin polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-huCr1 containing the human Osteoprotegerin cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22

were phophorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see diagram below) and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-20 #1257-19 (see above) was directionally inserted between two sites of the plasmid vector pAMG21, namely the NdeI site and BamHI site, using standard recominant DNA methodology (see diagram and sequences below). The synthetic linker utilized E. coli codons and provided for a N-terminal methionine.

Two clones were selected and plasmid DNA isolated, and the human Osteoprotegerin insert was subsequently DNA sequence confirmed. The resulting pAMG21 plasmid containing amino acids 32-401 of the human Osteoprotegerin polypeptide immediately preceeded in frame by a methionine is here to referred to as pAMG21-hu-Osteoprotegerin-32-401 or pAMG21-huCr1-32-401

Oligo#1257-19
5'-TACGCACTGGATCCTTATAAGCAGCTTATTTTTACTGATTGGAC-3'

Oligo#1257-20
5'-GTCCTCCTGGTACCTACCTAAAACAAC-3'

Oligo#1257-21
5'TATGGATGAAGAAACTTCTCATCAGCTGCTGTGTGATAAATGTCCGCC
GGGTAC-3'

Oligo#1257-22
5'CCGGCGGACATTTATCACACAGCAGCTGATGAGAAGTTTCTTCA
TCCA-3'

Cultures of pAMG21-hu-Osteoprotegerin-32-401 in E. coli GM120 in 2XYT media containing 20 ug/ml kanamycin were incubated at 30°C prior to induction. Induction of huCr1-co-DN10 gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by microscopy for the presence of inclusion bodies and were then pelletted by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant hu-Osteoprotegerin-32-401 gene product was produced insolubly in E. coli. Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemalli sample buffer to 1X final, and b-mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie stained band of approximately 42kDa was observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture. The expected gene product would be 370 amino acids in length and have an expected molecular weight of about 42.2 kDa. Following induction at 37°C for 6 hours, an additional culture was pelletted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing. The pellet processed for microfluidizing was resuspended in 25mM Tris-HCl / pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and

collected. An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder was pelletted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction). To an aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemalli sample buffer and -β mercaptoethanol to 5% final concentration. The samples were then analyzed by SDS-PAGE. A significant amount of recombinant hu-Osteoprotegerin-32-401 gene product appeared to be found in the insoluble fraction. To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and

distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion bodies were pelleted by centrifugation, and the protein concentration estimation following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemalli sample buffer + 5% b-mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant hu-Osteoprotegerin-32-401 gene product. The major ~42 kDa band observed after resolving inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira et al. J. Biol. Chem. 262, 10-35 (1987)). The following sequence was determined after 19 cycles:

NH2 -MYDEETSHQLLCDKCPPGT-COOH

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21-hu-osteoprotegerin-32-401 expression vector, produced by a methionine residue provided by the bacterial expression vector.

* * *

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.